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P. Capek, Maria Matulova, Bruno Combourieu

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P. Capek[a,*], M. Matulová[a], B. Combourieu[b]

[a] *Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia*
[b] *Laboratoire de Synthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504, Université Blaise Pascal - CNRS, 63177 Aubière cedex, France*

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A B S T R A C T

Highly viscous extracellular proteoglycan (EPG) has been isolated from culture medium of the unicellular red alga *Rhodella grisea* (Rhodophyceae) by ethanol precipitation. EPG was composed of xylose (29.3%), 3-*O*-methyl-xylose (26.0%), uronic acids (17.1%), rhamnose (14.4%), galactose (7.5%), glucose (3.9%), arabinose (1.4%) and mannose (0.4%), and traces of fucose, 4-*O*-methyl-xylose and 2,3-di-*O*-methyl-rhamnose or fucose. In addition, the polymer contained proteins (13.1%), sulphates and ^{13}C-CP MAS spectra indicated the presence of acetyl and succinyl groups. The molecular mass was estimated to be 136,000. Ion-exchange chromatography afforded five fractions differing in composition of neutral sugars, uronic acids, and protein content indicating thus the complex structure of the EPG.

1. Introduction

Algae are classified as the lowest plant organisms. They represent a diverse group of microscopic organisms, some of which can occur as aggregates of cells that contain chlorophyll *a* and carry out photosynthesis. Algae could be classified into individual algal divisions on the basis of several parameters e.g. the presence of phytopigments other than chlorophyll *a*, cell wall composition, presence or absence of flagella, type of polysaccharide storage, etc. [1]. Algae are cultivated mainly for the production of biopolymers possessing interesting rheological and biotechnological properties, with a wide range of various industrial (e.g. stabilizers, thickeners, emulsifiers in foods, etc.), pharmacological (drug delivery matrix) and medicinal (antiviral, hypocholesterolemic, antithrombotic, immunomodulatory activities, etc.) applications [2–7].

Unicellular red alga *Rhodella grisea*, previously described by Geitler (1970) as *Porphyridium griseum* (collected from Neusiedler See, Austria), was collected from Piešťany spa thermal water by Hindák [8]. Production of higher amounts of biopolymers by this alga was observed in comparison with other red algae. Individual *R. grisea* cells observed under the microscope are coated with a distinctive gelatinous polysaccharide matrix [8]. Thus when the alga was growing in the liquid medium it was producing very viscous culture medium. Up to date, no details are available about the type and composition of this complex proteoglycan. Consequently, the present study reports on its isolation and characterization.

* Corresponding author. Tel.: +421 2 59410209; fax: +421 2 59410222.
E-mail address: chemcape@savba.sk (P. Capek).

2. Material and methods

2.1. Cultivation of algae

The red alga *R. grisea* (strain Hindák, 1983) was grown with aeration (1% CO_2 in air) on a medium of Pekárková [9]. Extracellular proteoglycan (EPG) was isolated from *R. grisea* harvested at the stationary phase of growth. Algae cells were separated by centrifugation and biopolymer recovered from culture medium by ethanol precipitation and freeze-drying in 1988.

2.2. General methods

Solutions were concentrated under diminished pressure at a bath temperature below 40 °C. Optical rotation (1 mL cells) was measured at 20 °C with a PerkinElmer Model 141 polarimeter. Free-boundary electrophoresis of polysaccharide solutions was performed in 0.05 M sodium tetraborate with a Zeiss 35 apparatus at 150 V and 8 mA for 35 min. FT-IR spectra of polysaccharides were recorded with a Nicolet Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software. The sample was pressed into KBr pellet with a sample/KBr ratio 1/200 mg. HPLC analysis was performed with Shimadzu apparatus (Vienna, Austria) using a HEMA-BIO 1000 column of dimensions 8 mm × 250 mm and particle size 10 μm. As a mobile phase 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.8 mL/min. A set of pullulan standards was used for calibration of the column (Gearing Scientific, Polymer Lab. Ltd., UK). The amino acid composition was established with an Automatic amino analyzer, type 6020 (Mikrotechna, Prague), after hydrolysis of polysaccharide with 6 M HCl for 20 h at

100 °C. Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, UK). Protein was calculated from the nitrogen content (% N × 6.25). The methoxyl group content was determined according to the Vieböck–Schwappach method in a Zeisel apparatus [10].

Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. The quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography [11]. The uronic acid content was determined with the 3-hydroxybiphenyl reagent [12]. Gas chromatography–mass spectrometry of partially methylated alditol acetates was performed on a FINNIGAN MAT SSQ 710 spectrometer equipped with a SP 2330 column (0.25 mm × 30 m) at 80–240 °C, 70 eV, 200 A, and ion-source temperature 150 °C [13].

2.3. Hydrolysis and chromatography of hydrolyzed products

The extracellular proteoglycan (15 mg) was hydrolyzed with 2 M TFA (5 mL) at 120 °C for 1 h. TFA was evaporated and a part of the hydrolyzed mixture (~1 mg) was reduced and acetylated. The residue was subjected to the preparative paper chromatography by a descending method on Whatman 3 mm in the solvent system S, 8:2:1 ethyl acetate–pyridin–water. A stripe of the preparative paper was stained with anilinium hydrogenphthalate to identify sugar spots and after individual monosaccharides (nine fractions) were eluted with water, concentrated and freeze-dried.

2.4. Fractionation of EPG

The crude mixture of EPG (0.1 g) was dissolved in distilled water (200 mL) and applied to a column (4 × 15 cm) of DEAE-Sephacel and eluted successively with water, 0.1 M, 0.25 M, 0.5 M, 1 M NaCl, and 0.1 M sodium hydroxide solutions. Ten milliliter volume was collected and analyzed for the carbohydrate content by the phenol–sulphuric acid assay [14]. Pooled fractions were dialyzed and freeze-dried.

2.5. NMR spectroscopy

High-resolution solid state ^{13}C-CP MAS NMR was measured on a Bruker Avance DSX spectrometer operating at 75.46 MHz in a commercial Bruker double bearing probe in 4 mm ZrO_2 rotors. Acquisition of 2000 scans was performed at 10 kHz at room temperature using a VACP (variable amplitude cross polarization) sequence, a standard pulse program of Bruker library, with 3.3 μs proton 90° pulse, 1 ms contact time and 5 s relaxation delay. Chemical shifts were referenced to an external standard glycine (δ 176.03 ppm).

3. Results

Rhodella grisea at the stationary phase of growth was used for crude EPG isolation. Its molecular mass (M_w) was estimated to 138,000. The protein content and methoxyl groups suggested that the polymer is present in form of partly methylated proteoglycan. A low content of sulphur indicated the presence of sulphate groups (Table 1).

Monosaccharide analysis of the mixture, obtained after the hydrolysis of EPG, was performed by gas chromatography. On the basis of retention times it indicated the dominance of xylose, ribose and rhamnose, the presence of smaller amounts of galactose, glucose, arabinose, mannose and traces of fucose, suggesting thus a high heterogeneity of EPG. Paper chromatography, however, did not confirm the presence of ribose in the mixture but it has revealed, *inter alia*, the presence of four unknown sugars (US_{1-4}) having faster chromatographic mobilities than rhamnose. A red blob in two fractions US_{1-2} indicated the presence of pentose while a brown blob in US_{3-4} fractions hexose residue.

Table 1
Components identified in *R. grisea* extracellular proteoglycan (EPG)

Monosaccharide composition (%)		Amino acid	Mole (%)
Galactose	7.5	Glu	16.1
Glucose	3.9	Asp	14.3
Mannose	0.4	Ala	11.1
Arabinose	1.4	Gly	10.4
Xylose	29.3	Tyr	9.2
Fucose	tr.	Ser	7.6
Rhamnose	14.4	Leu	6.2
3-*O*-Methyl-xylose	26.0	Val	6.1
4-*O*-Methyl-xylose	tr.	Ile	4.7
2,3-Di-*O*-methyl-Rha/Fuc	tr.	Pro	3.7
Uronic acid	17.1	Phe	3.0
Methoxyl group (%)	5.8	Lys	2.7
Sulphur (%)	<0.5	Tyr	2.2
Nitrogen (%)	2.1	Arg	2.0
Protein (%)	13.1	His	0.7
Molecular mass (M_w)	138,000		
$[\alpha]_D$ (c 0.1, water)	−100°		

tr.: trace.

US_{1-4} fractions were converted into their acetate derivatives and thereafter identified by GC–MS as the 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-pentitol (US_1), 1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl-pentitol (US_2), and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-deoxy-hexitol (US_4). The structure of US_3 remained undetermined due to a very low yield and the complexity of the mass spectrum. Analysis of 1D and 2D NMR spectra of US_2 afforded detailed NMR data which were in consistency with α- and β-xylose substituted at O_3 by methyl group (data not shown). This fact suggests that its retention time should be the same as that one due to ribose. The same is valid for 4-*O*-methyl-xylose present in US_1. Analysis of other derivatives is in progress. Table 1 shows a list of components found in *R. grisea* EPG.

Due to an extremely high viscosity of the native proteoglycan it was not possible to acquire its ^{13}C NMR spectrum in water solution. However, a high-resolution solid state ^{13}C-CP MAS spectrum revealed more about the nature of sugars (uronic acid or deoxy-sugar), anomeric configuration of glycosidic linkages and possible types of substituents (Fig. 1). The presence a high content of uronic acids was confirmed by a large group of carbonyl signals at the lowest magnetic field (δ 176.5–173.4). Between them also carbonyl due to acetates should be present because of its CH_3 signal appeared at ~δ 21.0. The signal at δ 17.4 was attributed to C6 of rhamnose, while its anomeric C1 signal appears around δ 100.8. The most important signals in the spectrum are due to xylose and its *O*-methyl derivatives. Chemical shifts of their C1 signals indicate β-configuration. A broad hump at δ 85.7 contains signals due to carbon atoms of saccharide units downfield shifted due to their substitution by the methyl group. Signals due to methyl groups are represented by broad signal δ 55.8–49.8. Origin of the signal at δ 93.1 is not clear yet. Broad signal of low intensity at δ 29.9 suggests the presence of succinate.

The FT-IR spectrum of the native EPG (Fig. 2) showed the presence of bands characteristic of polysaccharides (1200–1000 cm^{-1}) and proteins (1641 and 1543 cm^{-1}). The main band at 1043 cm^{-1} is due to xylan moiety and the anomeric bands at 895 and 781 cm^{-1} suggest the β-configuration of xylose and α-configuration of rhamnose units, respectively. In addition, three bands at 1417 cm^{-1} ($\nu_s COO^-$), 1608 cm^{-1} ($\nu_{as} COO^-$), and 1726 cm^{-1} ν(C=O) characteristic for the carboxylic group of uronic acid were also present in the spectrum [15,16].

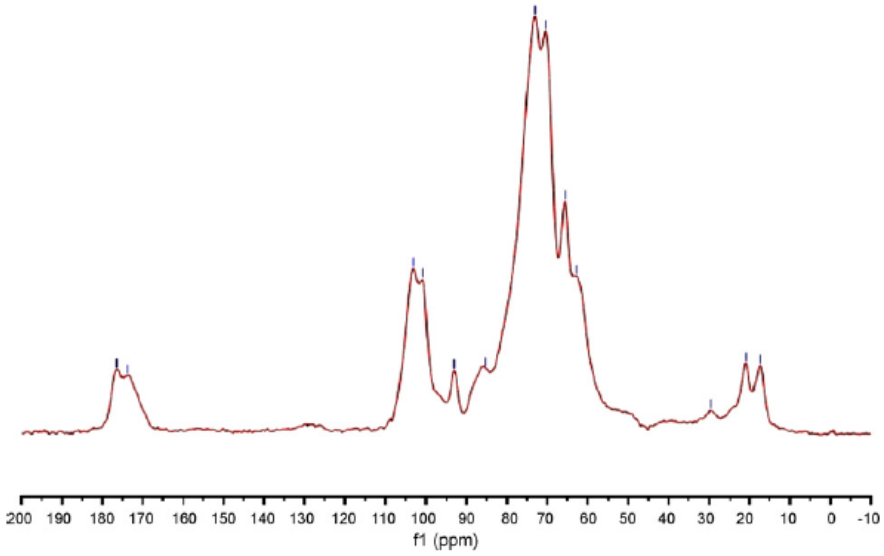

Fig. 1. ^{13}C-CP MAS spectrum of the native EPG.

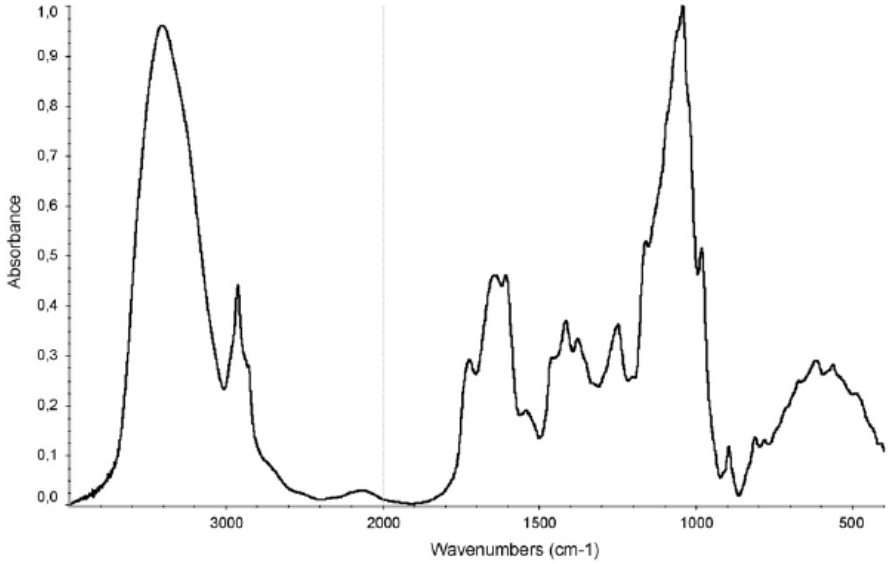

Fig. 2. The FT-IR spectrum of the native EPG.

Because of the monosaccharide analysis indicated the heterogeneity of the native EPG, ion-exchange chromatography was employed to verify its homogeneity. When water was used as eluent no carbohydrate material was eluted. However, a stepwise elution with 0.1–1.0 M sodium chloride and a final one with 0.1 M sodium hydroxide afforded five polysaccharide fractions confirming thus the heterogeneity of the EPG.

The preliminary results revealed an unusual type of the extracellular proteoglycan produced by *R. grisea*. Its carbohydrate part was composed of 57% pentose, 17% uronic acids, 14% rhamnose, and 12% hexose (galactose, glucose and mannose) residues only. The polymer appears to be a mixture of rhamified acidic xylans, highly acetylated, may be succinylated with a high content of 3-*O*-methyl-xylose, smaller amounts of 4-*O*-methyl-xylose and traces of 2,3-di-*O*-methyl-rhamnose/fucose. The high negative value of optical rotation, the chemical shift δ 103.2 of the dominant xylose residues in the ^{13}C-CP MAS spectrum, and the anomeric bands at 895 in FT-IR spectrum confirm the prevalence of β-type linkages in EPG. Its chemical composition significantly differs from extracellular polysaccharides produced by *Rhodella reticulata* [2] or related *Porphyridium* species [7].

Detailed structural characterization of the EPG produced by *R. grisea* is in progress and will be published in the following article.

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